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Food Quality and Safety Research,
National Center for Agricultural Utilization Research,
Agriculture Research Service/USDA, 1815 North University Street,
Peoria, Illinois 61604,
and Department of Animal Science, University of Illinois,
Urbana, Illinois 61801

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Food Quality and Safety Research, National Center for Agricultural Utilization Research, Agriculture Research Service/USDA, 1815 North University Street, Peoria, Illinois 61604, and Department of Animal Science, University of Illinois, Urbana, Illinois 61801

A supercritical fluid extraction (SFE) method using CO_2 has been developed for the removal analysis of fat from ground beef samples for nutritional analysis. The SFE procedure was coupled with a solid phase extraction (SPE) disk for the isolation of fats from acid hydrolyzed ground beef samples prior to SFE. The meat hydrolysate is filtered and collected on the reversed-phase SPE disk which is then subjected to SFE. The extracted fat is then transesterified to fatty acid methyl esters (FAMEs), and total, saturated, and *cis*-monounsaturated fats were determined by gas chromatography. The SFE method was tested on two commercial extractors and the results compared to a solvent-based (ether/hexane) extraction method. No significant differences were found between the results obtained by the solvent-based and the SC-CO_2 methods. The SFE method proved a suitable replacement for a traditional organic solvent extraction methods, thereby eliminating the use and costs associated with solvent disposal as well as the exposure of laboratory personnel to toxic and/or flammable solvents.

Keywords: *Supercritical fluid extraction; solid phase extraction; CO_2; lipid analysis; triglycerides; fatty acid methyl esters; ground beef*

INTRODUCTION

The Food and Drug Administration (FDA) regulation (*Fed. Regist.*, 1993a), implementing the Nutritional Labeling Education Act (NLEA) of 1990, defines total fat as the sum of all fatty acids obtained from a total lipid extract expressed as triglycerides. Although there is currently no method approved by the Association of Official Analytical Chemists International (AOAC) for fat determination utilizing this definition, current methods for the extraction of fats from meat samples generally require the use of a relatively large quantity of organic solvent, such as hexane or ether (e.g., House et al., 1994). Since the use of organic solvents is coming under increasing scrutiny due to their adverse environmental impact, and costs associated with both their purchase and disposal (Chester et al., 1994), the Environmental Protection Agency (EPA) has directed government agencies to reduce consumption of solvents in Federal laboratories (*Fed. Regist.*, 1993b). Consequently, our research group has been investigating analytical supercritical fluid extraction (SFE) as an alternative to solvent-based extraction methods. SFE, utilizing supercritical carbon dioxide (SC-CO_2), is gaining acceptance, due to carbon dioxide's relatively low toxicity and reasonable cost. Further, SFE methods employing SC-CO_2 are environmentally safe, resulting in reduced extraction times and automation with commercial instrumentation (Lehotay et al., 1995).

The NLEA definition and resultant methodology for speciated fat analysis is designed to remove some of the ambiguity associated with fat determinations. Many technical organizations specify particular analytical methods for determining fat which utilize a variety of sample preparation techniques, solvent types, and extraction methods; e.g., the AOAC lists over 30 different methods for determining fat in different sample matrices (Carpenter, 1992). Likewise, preextraction sample preparation techniques, such as hydrolysis of the sample matrix, use different types of chemicals and ionic strengths, incubation periods, and hydrolysis temperatures, however, hydrolytic treatment of food samples is dominated by concentrated acid hydrolysis.

Perhaps the key variable leading to inconsistent results in gravimetric-based fat assays is the wide variety of extraction solvents which have been utilized for extracting lipid matter from foodstuffs. Whereas nonpolar to intermediate polarity solvents, such as hexane, petroleum ether, diethyl ether, or acetone, yield approximately the same gravimetrically-derived value for fat content, the introduction of more polar solvents, such as chloroform:methanol (2:1) or ethanol, yield higher gravimetric fat results (Bligh and Dyer, 1959). The results obtained with higher polarity solvents are also partly due to the solubilization of more polar lipid species; however, these polar solvents also extract nonlipid moieties such as sugars, peptides, etc., albeit in small amounts, which influence the final result. The propensity of water to be extracted with these polar solvents also influences gravimetry-based measurements.

The aforementioned hydrolytic procedures are designed not only to partially cleave fatty acid moieties from lipid structures but to release "bound" lipid species (Inkpen and Quackenbush, 1969). This generally will result in an increase in the lipid matter ascertained by gravimetry. However, the hydrolysate is still subject to the limitations of the extraction technology cited above; e.g., the Folch (Folch et al., 1957) or Bligh–Dyer (Bligh and Dyer, 1959) methods, designed for extracting more polar lipid species, are still nonspecific with respect to other interfering compounds. Whereas many processed fats and oils are devoid of the above polar and

† Agriculture Research Service/USDA.
‡ University of Illinois.

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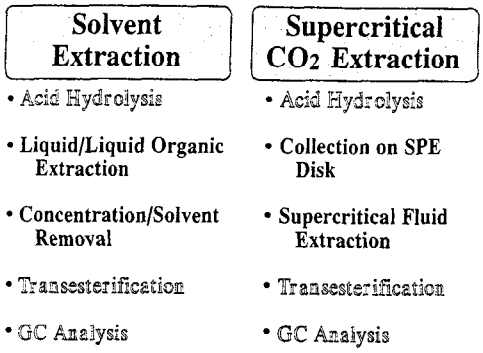

Figure 1. Comparison of the supercritical CO_2 extraction method and a solvent-based method for analysis of fats from meat.

bound lipids (Lumley and Colwell, 1991), fat derived from biological tissue, such as meats, will contain potentially some contribution from polar lipids (Maxwell, 1987).

Analytical SFE has been performed in our laboratory on a variety of food matrices, including meat samples ranging from 1.8 to 88% fat content (King, 1994) and snack foods containing 1.7−49.5% fat as determined by gravimetry. However, extensive preliminary SFE studies on ground turkey and beef samples have shown that fat values determined gravimetrically vary widely with extraction conditions. For example, SFE on the same lot of ground turkey meat gave gravimetric fat values ranging from 10.7 wt % (moist sample with neat CO_2) to 19.6 wt % (dehydrated sample extracted with SC-CO_2 using chloroform as a cosolvent) (King, 1994)! This was found to be true even on dehydrated samples (when the effect of sample water content on the variability of the analytical result was eliminated). Similar experiments performed with ground beef on a commercial SFE instrument showed a 42% increase in the fat content of the sample using gravimetry. However, when only SC-CO_2 was used to extract dehydrated ground beef samples, the gravimetrically-determined values were only slightly higher (0.5−0.6 wt %) than the results determined from SC-CO_2 extraction of samples which had been hydrolyzed and dehydrated and the results determined by fatty acid methyl ester (FAME) gas chromatographic (GC) analysis (King, 1994). This suggested that meaningful results could potentially be obtained by coupling SFE with preextraction hydrolysis of the sample, followed by FAME analysis which is specific for lipid moieties in the extracted hydrolysate.

Several researchers have found SFE using CO_2 to be a suitable replacement for traditional organic solvent extractions (Taylor et al., 1993; Lembke and Englehart, 1993; Hopper et al., 1995), particularly when comparing it with the results obtained with nonpolar solvents. The precision of fat analysis when performed by analytical SFE is comparable to the variance recorded when traditional organic solvent-based extraction protocols are used and, in many cases, is better than the standard deviations reported for liquid solvent extractions. The objective of this research was to develop a SFE method to replace current solvent-based extractions of fats from meat according to analysis mandated by NLEA/FDA regulations. This study describes a SC-CO_2 extraction method for fats from meat samples and compares it to a solvent-based method (as shown in Figure 1) which addresses the NLEA criteria for speciated fat analysis (House et al., 1994). Here, the SFE procedure is applied after acid hydrolysis of a weighed meat sample, and the fat present in the meat sample is adsorbed onto a reversed phase filter disk, which is then subsequently extracted with SC-CO_2 to remove the retained fat. The fat residue is then transesterified to yield the respective fatty acid methyl esters (FAMEs) and then analyzed by GC. Total fat is then calculated as the sum of all fatty acids, expressed as triglycerides, with the corresponding saturated and *cis*-monounsaturated fat content computed similarly from the sums of the appropriate FAMEs associated with these types of fat.

MATERIALS AND METHODS

Reagents. FAME standards were purchased from Nu-Chek Prep, Inc. (Elysian, MN). Boron trifluoride (14% BF_3 in methanol) was purchased from Alltech, Inc. (Deerfield, IL).

Ground Beef Samples. The ground beef samples were prepared by the Department of Animal Science at the University of Illinois, Urbana, IL. Ground beef samples with three nominal levels of fat content were prepared containing approximately 10, 20, and 30% fat. Beef trimmings were utilized and initially ground through a 13-mm plate and then mixed in a ribbon mixer for additional homogeneity. This substrate was then reground through a 3-mm plate, to yield a consistency typical of commercial ground hamburger, and then further homogenized in a bowl cutter assembly, to yield a very fine sample of pastelike consistency, which appeared homogeneous to the eye, lean and fat particles being indistinguishable. Approximately 75 lb sample lots of each ground beef sample were prepared for each of the above fat levels. The homogenized samples were then stuffed automatically into vacuum bags (ca. 125 g/bag), sealed, and frozen at −20 °C until further evaluation.

Acid Hydrolysis of Sample. Samples were digested by acid hydrolysis following the general procedure of House et al. (1994). A 1-mL aliquot of triundecanoin (C_{11} triglyceride) (Nu-Chek Prep, Elysian, MN) internal standard solution (5.000 mg/mL in chloroform) was pipetted into a 5-mL Erlenmeyer flask and the solvent evaporated under a gentle stream of nitrogen. Approximately 2 g of the sample was weighed (to the nearest 0.0001 g) into the flask, and ca. 100 mg of pyrogallol was added to prevent degradation of fatty acids during the hydrolysis. A 2-mL aliquot of ethanol and 10 mL of 8.0 N HCl were added to the flask and mixed well. The flask was stoppered and placed into a shaker bath (80 °C and 150 rpm) for 40 min and then cooled to room temperature.

Collection of Fat on Solid Phase Extraction (SPE) Disk. The cooled acid hydrolysis products were filtered through a 47-mm Empore Oil & Grease extraction disk (3M, Saint Paul, MN) with ca. 1 tsp of Empore Filter Aid 400 high-density glass beads (3M) on top of the disk using a 47-mm diameter microfiltration apparatus (Kontes Ultraware, Vineland, NJ). The extraction disk and glass beads were prewashed by rinsing with ca. 10 mL of hexane, dried for ca. 5 min, and wetted with ca. 10 mL of methanol. Methanol was then used to rinse the flask and the rinse put through the filter. The sides of the funnel were also rinsed with methanol and the filtrate dried for ca. 30 min.

SFE of Fat Collected on SPE Disk. After drying, the rolled-up disk and glass beads were placed in a 10-mL supercritical fluid extraction cell with glass wool plugs in each end of the cell. The supercritical extraction of the fat from the SPE disks was performed with neat SFC/SFE grade CO_2 (Air Products, Allentown, PA) using two commercial instruments: a Hewlett-Packard Model 7680T (Hewlett-Packard Co., Wilmington, DE) and an ISCO Model SFX 2-10 extractor using a Model 260D syringe pump (ISCO Inc., Lincoln, NE). The Hewlett-Packard 7680T utilized an automatic variable restrictor while the ISCO SFX 2-10 had a manual variable restrictor. Extraction parameters for both SFEs were as follows: 80 °C and 5500 psi (CO_2 density ca. 0.81 g/mL); a liquified CO_2 flow rate of 4 mL/min for 35 min (i.e., for a total of 140 mL of CO_2); and restrictor temperature of 50 °C. With the ISCO unit, the flow rate was read at the pump and adjusted by hand, as necessary, to maintain an approximate flow rate of 4 mL/min.

Both SFE units were used, bypassing their normal collection systems; the extracted fat being collected in 30-mL test tubes having Teflon caps. The transfer line from the restrictor to the collection vial on the 7680T, which is not present on the ISCO SFX 2-10 unit, was rinsed with 1 mL of HPLC grade hexane at the end of the extraction, and the hexane solution was evaporated under a gentle stream of nitrogen before transesterifying the fat.

Transesterification to Fatty Acid Methyl Esters. The collected fat was transesterified as described by House et al. (1994). The fat residue was dissolved in 1 mL of toluene and placed in a 12-mL screwcap vial with 1 mL of 7% BF_3 in methanol. The vial was then sealed and heated to 100 °C for 45 min with gentle shaking ca. every 10 min. The vial was removed from the oven and cooled to room temperature. A 5-mL aliquot of distilled water, 1 mL of hexane, and ca. 1 g of Na_2SO_4 was added to the vial and mixed vigorously. Two layers were allowed to form (centrifuged to speed separation), and the top layer removed and dried over ca. 1 g of anhydrous Na_2SO_4 in a separate vial. Completeness of FAME conversion was verified by supercritical fluid chromatographic analysis of the derivatized extract which showed only the presence of FAMEs and no triglycerides in the chromatographic profile.

Gas Chromatographic (GC) Analysis and Quantification of Fat. FAMEs were analyzed by GC according to the method of House et al. (1994). The FAMEs were analyzed by split injection (200:1 split ratio) onto a Hewlett-Packard Model 5890 series II gas chromatograph (GC) equipped with a flame ionization detector. The column used for analyzing the FAME derivatives was a SP-2340 (60 m; 0.25-mm diameter; 0.20-μm film thickness) (Supelco Inc., Bellefonte, PA), and the carrier gas was He, utilizing a linear flow velocity of 18 cm/s through the column. The temperature programmed run was 100 °C for 5 min, 3 °C/min to 190 °C, 1 °C/min to 200 °C and held for 15 min, 50 °C/min to 250 °C and held for 1 min. The injector and detector temperatures were 235 and 250 °C, respectively. Injections were made using a Hewlett-Packard 7673 auto injector, and the sample volume injected was 1 μL. The chromatographic data were acquired using a Hewlett-Packard Vectra VL2 computer and ChemStation software. The weights of the individual FAMEs were calculated on the basis of their integrations relative to that of the triundecanoin internal standard and were corrected using respective GC response factors. The weights of the individual FAMEs were converted to equivalent weights of triglycerides by appropriate conversion factors (Carpenter et al., 1993). Total fat was calculated as the sum of all fatty acids obtained from a total lipid extract expressed as triglycerides. Saturated and *cis*-monounsaturated fats were also similarly calculated from the sums of the saturated and *cis*-monounsaturated fatty acids, respectively.

Solvent-Based Extraction of Samples. Ground beef samples were sent to a commercial laboratory (Medallion Laboratories, Minneapolis, MN) to be analyzed by a solvent-based method for comparison to the SFE method. The fats were collected by liquid–liquid extraction of the acid hydrolysate using ether and hexane (House et al., 1994). The acid hydrolysis, transesterification of the collected fats, and GC analysis of FAMEs in this case were the same as reported for the SFE method.

Statistical Analyses. Three replicate analyses were performed on each sample type–extraction method combination. Analyses of variance (ANOVAs) were performed on the calculated percent total fat, saturated fat, and *cis*-monounsaturated fat using Statistix 4.1 software (Analytical Software, Tallahassee, FL).

RESULTS AND DISCUSSION

In the development of a method involving the substitution of analytical SFE for the organic solvent extraction of fats from meats, it was essential to approach the problem systematically. Key steps in the development process were verification of the homogeneity of the sample matrix, initial optimization of the extraction method, transfer and modification of the initial extraction conditions to commercial instrumentation, and proving equivalence of the SFE-based method with the solvent-based method. To test the homogeneity of the prepared meat samples, four subsamples were taken of an individual ground beef packet, dissected into quarters, and analyzed by an identical procedure to the one described above, using the conventional organic solvent extraction protocol (House et al., 1994), by an independent laboratory (Medallion Laboratories). These results are presented in Table 1 along with the average for the total, saturated, and *cis*-monounsaturated fat content. As indicated by associated relative standard deviations for each type of fat, there is little variance in the speciated fat content within the ground beef packet. This trend was also found to hold when identical extractions and analysis on other ground beef packets of this initial ground beef sample were performed. These results indicated that the method for preparing the ground beef samples yielded a homogeneous sample matrix that could be used with confidence in subsequent extraction studies.

Table 1. Percent Total, Saturated, and *cis*-Monounsaturated Fat Content in Subsamples of Ground Beef[a]

subsample no.	type of fat		
	total	saturated	*cis*-monounsaturated
1	14.7	6.0	6.5
2	14.9	6.1	6.6
3	14.4	5.9	6.4
4	14.5	5.9	6.4
av (RSD)	14.6 (1.5)	6.0 (1.6)	6.5 (1.5)

[a] Fat content determined by GC-FAME analysis of ether/hexane extract after acid hydrolysis of sample.

Some initial experiments previously reported (King, 1994), indicated that SFE performed on acid hydrolyzed 2-g meat samples using extraction conditions of 10 000 psi and 80 °C gave similar results to those obtained from the method using solvent extraction (King, 1994), followed by GC-FAME analysis of the resultant fat extract. For example, four separate extractions of the described ground beef packets yielded average values of 14.9, 6.4, and 7.5 wt % for the total, saturated, and *cis*-monounsaturated fat content, respectively. Although the relative standard deviations associated with these averages were 3.3, 3.1, and 2.6, respectively, somewhat higher than those recorded for the conventional solvent extraction procedure, the agreement between the means indicated that SFE could be used as a potential substitute for organic solvent extraction of fat from meat matrices.

Transfer of the method to commercial SFE equipment required optimization of the extraction parameters be consistent with the lowest extraction pressure available on commercial SFE equipment. This was judged critical since the described method would eventually be corroborated via a collaborative study by chemists using a variety of equipment. Hence, the conditions described in Materials and Methods in which the SFE was conducted at 5500 psi, 80 °C, etc., were found to be satisfactory as will be described shortly. Although the solubility of many lipid species is substantially decreased by reducing the pressure from 10 000 to 5500 psi (Stahl et al., 1988), this pressure was found to be sufficient for quantitative lipid extraction of the sample sizes noted above.

The mean (±SD) percent total fat, saturated fat, and *cis*-monounsaturated fat for the three ground beef

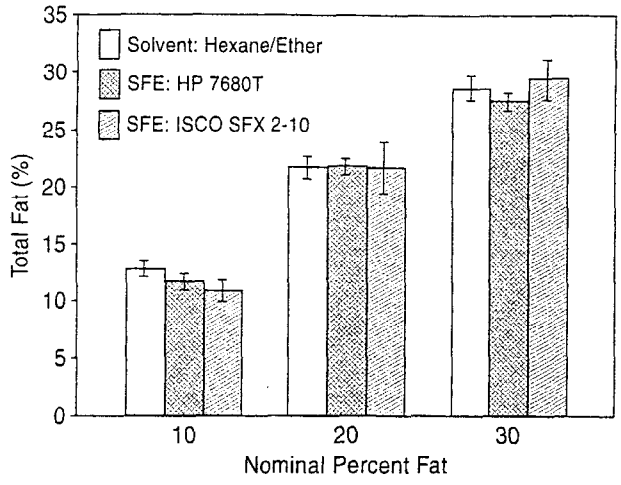

Figure 2. Mean (±SD) percent total fat for the three ground beef samples and the three extractions.

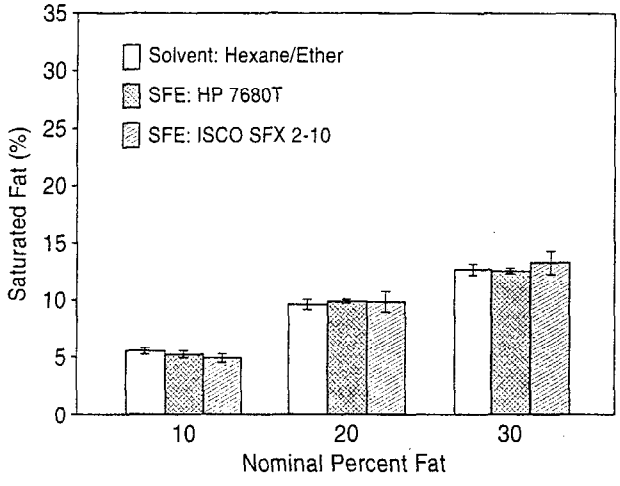

Figure 3. Mean (±SD) percent saturated fat for the three ground beef samples and the three extractions.

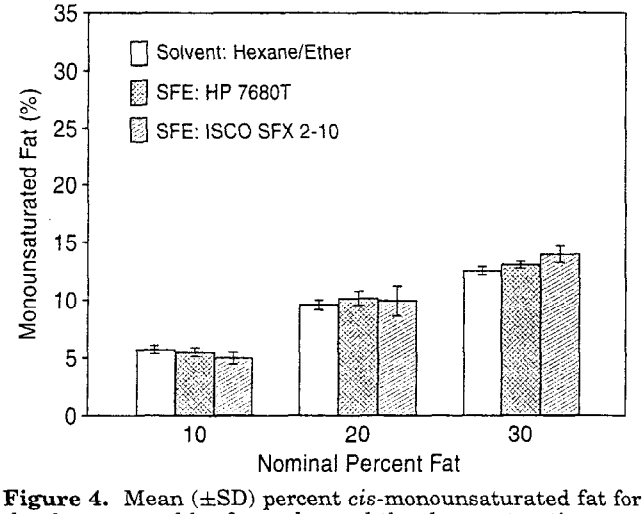

Figure 4. Mean (±SD) percent *cis*-monounsaturated fat for the three ground beef samples and the three extractions.

samples containing different levels of fat, extracted in triplicate, are shown as bar graphs in Figures 2–4, respectively. Because the variability of the data obtained from the ISCO extractor (for all types of fat) appeared higher than the results obtained from the other extractions, pooled variances for the three extraction methods were compared using Fisher's *F*-test (Snedecor and Cochran, 1967). Although the variance for the ISCO extractor was numerically higher than the variances for the other two extractions, partly due to the need to manually adjust the flow rate, it was statistically higher in only one of six possible pairwise comparisons (i.e., saturated fat: ISCO vs HP 7680) (Table 2).

Table 2. Pooled Variances for Extraction Methods[a]

	type of fat		
extracn method	total	saturated	*cis*-mono-unsaturated
ether/hexane (Medallion Laboratory)	0.88 a	0.18 ab	0.14 a
supercritical CO_2			
HP-7680T	0.54 a	0.06 a	0.20 a
ISCO SFX 2-10	3.10 a	0.68 b	0.83 a

[a] Variances within a column (i.e., type of fat) without letters in common differ significantly using Fisher's *F*-test for variances (Snedecor and Cochran, 1967).

As expected, the ANOVA of the percent total fat data indicated that there was a highly significant effect of beef sample ($F_{2,18df} = 1284.06$, $P = 0.00$) (i.e., the beef samples containing the three levels of fat were significantly different). There was no significant effect of extraction method ($F_{2,18df} = 0.66$, $P = 0.53$) nor was there a significant extraction method by beef sample interaction ($F_{4,18df} = 1.54$, $P = 0.23$) on the percent total fat. The mean percents of total fat for the three sets of extractions (i.e., solvent, HP-7680T, and ISCO SFX 2-10) were 21.1, 20.7, and 20.4, respectively.

The ANOVA of the percent saturated fat data also indicated that there was a highly significant effect of beef sample ($F_{2,18df} = 432.74$, $P = 0.00$). There was no significant effect of extraction method ($F_{2,18df} = 0.15$, $P = 0.87$) nor was there a significant extraction method by beef sample interaction ($F_{4,18df} = 1.29$, $P = 0.31$) on the percent saturated fat. The mean percents of saturated fat for the three sets of extraction methods (i.e., solvent, HP-7680T, and ISCO SFX 2-10) were 9.4, 9.3, and 9.2, respectively.

The ANOVA of the percent *cis*-monounsaturated fat data indicated that there was a highly significant effect of beef sample ($F_{2,18df} = 353.71$, $P = 0.00$). There was no significant effect of extraction method ($F_{2,18df} = 0.80$, $P = 0.46$) nor was there a significant extraction method by beef sample interaction ($F_{4,18df} = 2.27$, $P = 0.10$) on the percent *cis*-monounsaturated fat. The mean percents of *cis*-monounsaturated fat for the three sets of extraction methods (i.e., solvent, HP-7680T, and ISCO SFX 2-10) were 9.6, 9.6, and 9.3, respectively. Hence, the two SFE methods studied gave results equivalent to those from the standard organic solvent extraction method.

Although Lembke and Engelhardt (1993) digested meat samples by acid hydrolysis and collected the fat prior to SFE on a conventional folded filter paper our experience in attempting this method indicated that the fats were not adequately retained on the filter paper and that the filter paper leaked and lacked sufficient mechanical rigidity to permit reproducible insertion into the extraction vessel. The use of a SPE disk overcame these difficulties and allowed the quantitative extraction of fat without liquid–liquid solvent extraction of the acidified solution (Hedrick and Taylor, 1989, 1990). Other investigators have used SPE disks prior to SFE to isolate the target analytes. Howard and Taylor (1992) describe a somewhat similar approach where the analyte (a sulfonyl urea herbicide) was concentrated (from water) on a disk prior to SFE removal of the analyte from the disk. Liu et al. (1992) used a combined

SPE-SFE procedure to isolate drugs from a plasma matrix, while Hawthorne et al. (1992) used SPE-SFE to isolate phenols.

In conclusion, this study demonstrates the potential of using SFE with neat CO_2 for the replacement of organic solvents for the isolation of fat from ground beef samples according to the new NLEA protocol. SFE proved further to be effective for the extraction of fats retained on a SPE disk, and the combination of SPE with SFE was equivalent to hexane/ether extraction with respect to analysis accuracy and precision. The method has been adapted for use with several commercial extractors as well as laboratory constructed extraction equipment, and equivalent results have been obtained for speciated fat analysis. Further research is being conducted to substitute enzymatic hydrolysis followed by transesterification to form FAMEs from the extracted fat to permit on-line SFE analysis of fat in meat samples. However, the method described in this study will be subjected in the near future to a collaborative study and, hopefully, will result in reducing analysts dependence on organic-based extraction solvents in the laboratory environment.

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